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PRINCIPAL INVESTIGATOR: Fu-Ming Chen, Ph.D.

CONTRACTING ORGANIZATION: Tennessee State University
Nashville, TN 37209-1561

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Fu-Ming Chen, Ph.D.

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Proposal was made to study the sequence specific binding and synergistic effect of three drugs having distinctly different binding modes: actinomycin D (ACTD), a guanine specific intercalator; chromomycin A_3 (CHR), a guanine specific minor groove binder; and distamycin A, an A•T specific groove binder. To investigate the possible synergistic effects of drugs on DNA binding, it is essential that binding characteristics of each individual drug such as binding affinities, sequence specificities, and kinetic behaviors be well understood. During the past year, our laboratory has focused mainly on the detailed studies of CHR and its sequence specificity at the tetranucleotide level. Comparative studies with self-complementary decamers of the form d(GTA-XGCY-TAC) suggest that CHR binds strongly to a 5'GC3' site but the binding affinities and dissociation kinetic behaviors are greatly affected by the adjacent base pairs. For example, the CHR binding affinity and the dissociation rate at the -GGCC- site are an order of magnitude stronger and slower than those at the -AGCT- site. Studies were also extended to include non-self-complementary tetranucleotide -XGCY- sequences with decamers of the form d(GTA-XGCY-ATG)/d(CAT-Y'GCX'-TAC), where X' and Y' are complementary to X and Y, respectively. Again significant modulation by the adjacent base pairs on the binding and kinetic behaviors are seen.

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Ju-Ki Chen 10-21-96

PI - Signature Date

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INTRODUCTION

Combination chemotherapy is one of the important strategies in cancer treatments. This is based on the observation that administering certain drugs together is more effective than giving individual drugs separately. Although the reason for such an effect is not understood, it may be related to the synergistic effect of their binding to macrobiomolecules. Consequently, studies on the interplay among drugs capable of binding to different regions of DNA will be of considerable interest. Understanding the synergism of drugs at the molecular level may have important implication for designing more effective chemotherapeutic strategies in breast cancer treatments. Our proposal focuses on sequence specific binding and synergistic effect of three drugs having distinctly different binding modes: actinomycin D (ACTD), a guanine specific intercalator; chromomycin A_3 (CHR), a guanine specific minor groove binder; and distamycin A, an A•T specific groove binder. In order to investigate the possible synergistic effects of drugs on DNA binding, it is essential that binding characteristics of each individual drug such as binding affinities, sequence specificities, and kinetic behaviors be thoroughly elucidated. During the past year, our laboratory has focused on the detailed studies of CHR.

Chromomycin A_3 (CHR), mithramycin (MTR), and olivomycin are three closely related antitumor antibiotics of the aureolic acid class which contain an aglycon chromophore with di- and trisaccharide (2,6-dideoxyhexapyranose) side chains attached on opposite sides. The antitumor activity of these drugs is believed to be the consequence of the ability to bind to duplex DNA which results in the inhibition of DNA-directed RNA sythesis. these drugs are known to be guanine-specific (Ward et al., 1965; Kaziro & Kamiyama, 1967), and their DNA binding requires the presence of divalent cations such as magnesium (Behr & Hartmann, 1965; Goldberg & Friedman, 1971). Footprinting studies (Van Dyke & Dervan, 1983; Fox & Howarth, 1985; Con & Fox, 1989a,b; Stankus et al., 1992) have confirmed their guanine specificity and further indicated that the binding sites are at least three base pairs long. The presence of two contiguous G•C base pairs is neccessary but not sufficient by themselves for strong DNA binding of these aureolic acids. Consequently, their affinities appear to be modulated by flanking sequences.

Earlier physiochemical studies have produced contradictory conclusions on the DNA binding mode of these drugs. The general consensus was that nonintercalative binding occurred at the minor groove because CHR and MTR fail to affect some properties of DNA such as its sedimentation coefficient and viscosity (Goldberg & Friedman, 1971; Kersten et al., 1966) and do not significantly affect the supercoiling of DNA (Waring, 1970). Conversely, other reports indicated an intercalative (Prasad & Nayak, 1976; Dasgupta et al., 1979) or major groove (Keniry et al., 1987) binding mode. Recent definitive NMR studies on the CHR-d(TTGGCCAA) (Gao & Patel, 1989; Gao et al., 1992) and CHR-d(AAGGCCTT) (Gao & Patel, 1990) complexes in Mg^{++}-containing solutions have established unequivocally that CHR binds as a Mg^{++}-coordinated dimer at the minor groove of the central GGCC segment of the duplex and have revealed the structural basis for the sequence specificity at the 5'GC3' step. The minor groove drug

dimer binding model and sequence preference of GC over CG were subsequently verified by other NMR studies (Banville et al., 1990).

Although footprinting experiments have provided some sequence preference information on these drugs, no systematic investigation using other techniques to determine sequence specificity has been performed to date. Our earlier studies with polynucleotides have indicated some sequence or conformational effects on their DNA binding. For example, poly(dA-dG)•poly(dC-dT) exhibits stronger affinity for CHR and MTR than its sequence isomer poly(dA-dC)•poly(dG-dT) (Sarker & Chen, 1989). The detailed structural information available on the binding to -GGCC- (Gao et al., 1992) and -CGCG- sequences (Sastry & Patel, 1993) has provided considerable insight into the structure and dynamics of aureolic acid-DNA interactions. Hence, a systematic study on the specificities to other sequences via synthetic oligonucleotides would be of value.

During the past year, consistent with Task 2 of SOW, our focus has been on the sequence specific binding studies of CHR at the self- and non-self-complementary tetranucleotide level. Binding and kinetic studies were made with non-self-complementary decameric duplexes of the form d(GTA-XGCY-ATG)/d(CAT-Y'GCX'-TAC) and their results were compared with the self-complementary decamers of the forms d(GTA-XGCY-TAC) and d(GTA-XCGY-TAC).

BODY

Experimental Methods

As a first step in elucidating the tetranucleotide sequence specificity of aureolic acids, CHR binding and kinetic studies were carried out on self-complementary decamers of the form d(GTA-XGCY-TAC) and d(GTA-XCGY-TAC), where X = G, C, A, or T and Y is complementary to X. Studies were then extended to investigate the effects of adjacent base pairs which are not necessarily self-complementary, using oligomers of the form d(GTA-XGCY-ATG)/d(CAT-Y'GCX'-TAC), where X, Y = G, C, A, or T and X' and Y' are complementary to X and Y, respectively.

Equilibrium Binding Affinities. CHR exhibits an absorbance maximum at 406 nm when free in solutions. Successive additions of DNA lead to bathochromic shifts and intensity enhancements at 440 nm and a concomitant reduction at the 400-nm region. An isosbestic point is evident near 411 nm, suggesting a two-component process. Thus, absorbance changes at a given wavelength or a absorbance differences at two wavelengths can be used to construct binding isotherms and to obtain binding parameters. Absorbance differences at 440 and 400 nm were used to obtain binding isotherms and Scatchard plots. Since most of the Scatchard plots can be approximated by straight lines, the binding parameters were deduced via linear least-squares fits. A qualitative ranking of the relative binding affinities of CHR for different sequences can also be achieved by simply comparing the intensity enhancements of CHR absorbance around 440 nm in the presence of various oligomers of the same concentrations.

DNA melting measurements were also carried out to investigate the effect of CHR binding on the DNA duplex stability. The extent of melting temperature increase of DNA upon drug binding can also be correlated to the relative binding affinity.

Absorption spectra were measured with a Cary 1E spectrophotometric system. Spectral titrations were carried out at 25 °C by starting with a drug solution followed by progressive additions of the oligomer stock. Thermal denaturation experiments of 40 μM oligomer in the absence and in the presence of 5 μM CHR were carried out with 1-cm semimicro cells by monitoring the absorbance at 275 nm. A heating rate of 0.5 °C/min was maintained by the temperature controller accessory. Numerical differentiations were performed to obtain differential melting profiles from which melting temperatures were deduced.

CHR is optically active and exhibits negative and positive CD maxima at 295 and 276 nm, respectively, when free in solutions. In the presence of DNA, however, a strong excition-type couplet, presumably the result of dimer formation and/or aglycon-DNA base interactions, is induced which results in an inverted CD spectrum with positive and negative maxima appearing at 287 and 275 nm, respectively. Thus, induced CD intensities at 287 and 275 nm can also serve as gauges for the relative binding affinities of CHR for these oligomers. CD spectra were measured at room temperature by a Jasco J-500A recording spectropolarimeter using water-jacketed cylindrical cells of 1-cm path length.

Association and Dissocaition Kinetic Measurements. Association kinetic measurements were carried out at 25 °C by adding the DNA stock to the CHR solutions to result in drug and DNA concentrations of 4.85 and 40 μM, respectively. Time-dependent absorbance changes at 440 nm were monitored. The observed data can be adequately approximated by single-exponential decays, since the two-exponential fits did not result in significant improvements in the sum of square deviations. Dissociation kinetics were measured by adding 20% sodium dodecyl sulfate (SDS) stocks to the CHR-DNA solutions to result in 1% SDS. Time-dependent absorbances at 345 nm were monitored to obtain kinetic profiles. Dissociation rate constants were obtained via 2-exponential nonlinear least-squares fits since they provide significantly better fits than those of single-exponential.

Electrohporetic Evidence on CHR-DNA Complex Formation. Gel electrophoretic mobilities of oligomers in the absence and in the presence of CHR were also measured to provide evidence on drug-DNA complex formation. Comparison of the retarded band intensities can provide information on relative binding affinities and dissociation rates. Electrophoretic measurements were made on a Pharmacia Phast System using 20% polyacrylamide gels at 200 V with appropriate pre- and post-loading run times at different temperatures. PhastGel native buffer strips containing 0.25 M Tris of pH 8.8 were used, and the gels were developed by silver staining.

Results

CHR Binding to Self-Complementary Tetranucleotide Sequences. Comparison of absorption difference spectra, drug-induced CD difference spectra, melting temperatures, and binding parameters extracted via Scatchard plots (see Table 1) with the self-complementary decamers of the form d(GTA-XGCY-TAC) and d(GTA-XCGY-TAC) established the following CHR binding order: -GGCC- > -CGCG-> -GCGC-, -CCGG- > -AGCT- > -ACGT-, -TGCA- > -TCGA-.

The single-exponentially fitted association rate constants k_a and the double-exponentially fitted dissociation rate constants are also included in Table 1. As is apparent, no significant difference in the association rates were observed among the oligomers studied, and they range from 0.6 to 0.8 min^{-1}. Contrasting the association kinetics, dramatic variation in the rates of drug dissociation from oligomers is apparent. Strikingly slow SDS-induced CHR dissociation from the -GGCC- and -CGCG- olgomers, especially that of the former, were observed which are nearly an order of magnitude slower than from -GCGC- and -CCGG-. Also noteworthy is the fact that CHR not only dissociates from -AGCT- more slowly than from -ACGT- but also dissociated significantly slower than from -TGCA-. The trend exhibited by the dissociation rates is consistent with the suggested binding order established via equilibrium binding studies.

All four decamers containing four central G•C base pairs exhibited retarded bands upon drug complexation. The intensities of the retarded bands for the -GCGC- and -CCGG- oligomers, however, are considerably less intense than those of -GGCC- and -CGCG-. These results are consistent with their weaker binding and faster dissociation rates. For the decamers containing only two central G•C base pairs, only the -AGCT- oligomer exhibits a visible retarded band but its intensity is somewhat weaker than those of -GCGC- and -CCGG-. This is consistent with the significant CHR binding affinity of the -AGCT- sequence and the still weaker binding affinities of the other three oligomers.

CHR Binding to Non-Self-Complementary Decamers. To further investigate the effects of adjacent base pairs on the CHR binding behaviors, a more systematic study was carried out with decamers of the form d(GTA-XGCY-ATG) which were annealed with the corresponding complementary strands of the form d(CAT-Y'GCX'-TAC), where X, Y = G, C, A, or T and X', Y' are complementary to X, Y, respectively. Binding and kinetic parameters for these oligomers are summarized in Table 2. Due to the relocation of my full-time research assistant to Philadelphia in July and the coming on board only in mid-October of a new assistant, this study is currently in the process of being completed as evidenced by some missing data in the table.

Despite the incomplete study, some general patterns appear to be emerging: (1) rates of CHR association at the GC sites appear to be rather insensitive to the adjacent base pairs; (2) binding affinities and rates of dissociation are significantly affected by the adjacent base pairs; (3) in general the -XGCY- sites with X = G and/or Y = C exhibit stronger binding and slower dissociation rates; and (4) -GGCC- exhibits the strongest CHR binding affinity and the slowest dissociation kinetcs. These results are consistent with those of self-complementary decamers.

Comparison of CHR and MTR. Some studies were also made to compare the binding and kinetic behaviors of two very similar drugs, CHR and MTR, using self-complementary dodecamers of the form d(ATTA-XGCY-TAAT) and the results are summarized in Table 3. Interesting differences were observed: (1) MTR exhibits considerably weaker binding affinities towards -GGCC- and -CGCG- than CHR; (2) the rate of MTR dissociation from -GGCC- is more than an order of magnitude faster than that of CHR; (3) in contrast to the much stronger CHR binding for -GGCC- and -CGCG- as compared to -AGCT- and -TGCA-, MTR binds strongest to -AGCT-; and (4) instead of -GGCC-, the sequence -AGCT- exhibits the slowest MTR dissociation rate.

Discussion

Absorption and CD spectral evidence indicates that the CHR binding affinities of the self-complementary tetranucleotide sequences containing at least two contiguous G•C base pairs are in the following order: -GGCC- > -CGCG- > -GCGC-, -CCGG- > -AGCT- > -ACGT-, -TGCA- > -TCGA-. The general relative binding order is supported by gel retardation, thermal denaturation, and dissociation kinetic measurements. The SDS-induced drug dissociation from the -GGCC- and -CGCG-containing oligomers is found to be more than an order of magnitude slower than the rest of the sequences studied. The considerably weaker binding of -GCGC-, -CCGG-, and -ACGT, each containing a central 5'CG3' step, as compared to the corresponding sequence isomers -GGCC-, -CGCG-, and -AGCT-, each containing a central 5'GC3' step, is consistent with the preference of CHR to the GC step. However, CHR binding is seen to be strongly modulated by the adjacent base pairs or sequences. For example, despite the presence of a central GC step, CHR binding to -AGCT- is significantly weaker and dissociation occurs considerably faster than at -GGCC- and -CGCG-. This is further exemplified by -TGCA- which exhibits a much weaker affinity for the drug.

Thus, our results support the earlier NMR findings (Gao & Patel, 1989; Gao et al., 1992) which suggest the sequence specific binding of CHR to the 5'GC3' sequence. The strong preference for the GC step has been attributed to the head to tail dimer orientation of CHR at the widened and shallower minor groove binding site and is associated with specific intermolecular hydrogen bond formation of the OH group at the C8 of aglycon with NH_2 and N3 of guanine. Furthermore, the stronger bindig of -GGCC- and -CGCG- as compared to -AGCT- may be understood in terms of the additional hydrogen bonding capability of the E sugar ring with the 2-amino group of the guanine adjacent to the central GC step (Gao et al., 1992). The much weaker binding of -TGCA- as compared to -AGCT-, however, is not clear and may have its origin in the sequence-dependent conformational differences (such as groove width) and/or sugar rings and minor groove interactions.

Our finding of large variation in dissociation rates among different sequences is significant. The fact that CHR dissociates from -GGCC- significantly slower than from -CGCG- which in turn is more than an order of magnitude slower than from -AGCT- and -TGCA- suggests that G•C base pairs adjacent to the strong binding GC step may be involved in hydrogen bonding and are responsible for the observed much slower dissociation kinetics. As mentioned earlier, such hydrogen bonds were in fact observed in the CHR-d(AAGGCCTT) system (Gao et al., 1992) between E pyranose rings and the adjacent G•C base pairs. Leroy et al. (1991) recently extended the CHR-d(AAGGCCTT) structural studies to include hydrogen exchange measurements. It was found that the opening rate of the four central base pairs, which interact with CHR in the complex, is less than that of the free duplex by 1-2 orders of magnitude.

It is also interesting to compare the dissociation kinetics with those of actinomycin D (ACTD) which is also GC sequence specific but is intercalative in nature. For example, the SDS-induced CHR dissociation from d(GTA-GGCC-TAC) exhibits a characteristic time of more than 50 min at 25 °C which is comparable to the single characteristic time of 55 min for ACTD dissociation from d(ATA-TGCA-TAT) at 18.5 °C (Chen, 1988). The

slow dissociation kinetics exhibited by both drugs are no doubt related to the abilities of both chromophores to hydrogen bond to the central G•C base pairs and favorable side chain interactions with the minor groove. In contrast to the hydrogen bonding abilities of pyranose side chains of CHR, the pentapeptide ring interactions of ACTD with the minor groove are more hydrophobic in nature. This resulted in the preference of A•T adjacent base pairs, with -TGCA- becoming the strongest binding and slowest dissociation sequence (Chen, 1988). The strong dependence of dissociation rates on binding sequences also suggests that the observed multiple-exponential dissociation kinetics of CHR from native DNA (Behr et al., 1969) may likely be adequately described by the site-heterogeneity model, similar to those of ACTD (Krugh et al., 1980).

Although no significant variation in the rates of association was found among the sequences studied, the observed rate constants of 0.6-0.8 min^{-1} are worth commenting on. The characteristic association times of more than 1 min are considerably slower than for most other drugs. Such slow processes may originate from the requirement for widening of the minor groove to accommodate the bulky drug dimer and properly positioning the chromophores and side chains for optimal interactions.

A recent single-crystal X-ray analysis of d(CATGGCCATG) has revealed a smooth bend of 23 $^{\circ}$ over the central four base pairs in a direction that compresses the wide major groove of the double helix (Goodsell et al., 1993). As -GGCC- has been found to be the strongest CHR binding and the slowest dissociation site, it is tempting to speculate that such a bend may have resulted in a better fit for the Mg^{++}-mediated drug dimer in the minor groove of this particular sequence. The stronger binding and slower dissociation from non-self-complementary sites of -XGCY- with X = G or Y = C may likely be also conformational in origin. Our finding of strong MTR preference for -AGCT-, in contrast to -GGCC- for CHR, may likely be the consequence of more favorable side-chain interactions with the DNA, as these two drugs differ only in the side-chain placements.

CONCLUSION

Our experimental results support earlier NMR findings by other researchers implicating the preference of aureolic drugs at the 5'GC3' step and further reveal significant modulations by the adjacent base pairs. These results on sequence specificities provide a good foundation for our future study on the synergistic effects of drugs. Our next step will be to carry out similar sequence specificity studies on the A•T specific drug distamycin. The results of these studies on individual drugs will then form the bases for designing the study on synergism.

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Table 1. Summary of CHR Binding and Kinetic Parameters for Self-complementary Decamers at 25 °C.

Oligomers	K (μM^{-1})	*n*/dpx	k_a (min^{-1})	k_{1d} (min^{-1})	k_{2d} (min^{-1})	%
d(GTA-GGCC-TAC)	7.0	2.5	0.61	0.033 ± 0.001	0.0032 ± 0.0001	57
d(GTA-CGCG-TAC)	3.3	2.6	0.67	0.058 ± 0.001	0.0113 ± 0.002	94
d(GTA-GCGC-TAC)	1.0	2.7	0.69	1.78 ± 0.04	0.229 ± 0.003	70
d(GTA-CCGG-TAC)	1.0	2.9	0.57	1.82 ± 0.07	0.180 ± 0.003	79
d(GTA-AGCT-TAC)	0.6	2.4	0.77	0.42 ± 0.01	-	-
d(GTA-TGCA-TAC)	< 0.5	-	0.74	2.09 ± 0.06	0.185 ± 0.004	69
d(GTA-ACGT-TAC)	< 0.5	-	0.80	3.06 ± 0.01	0.27 ± 0.02	58
d(GTA-TCGA-TAC)	< 0.1	-	0.74	-	-	-

Table 2. Comparison of CHR Binding and Kinetic Parameters for non-self-complementary Decamers at 25 °C.

Oligomers*	K (μM^{-1})	*n*/dpx	k_a (min^{-1})	k_{1d} (min^{-1})	k_{2d} (min^{-1})	%
d(GTA-GGCG-ATG)	10.7	2.7	0.49	0.17 ± 0.03	0.015 ± 0.003	84
d(GTA-GGCC-ATG)	15.2	2.2	0.61	0.19 ± 0.02	0.0078 ± 0.001	91
d(GTA-GGCA-ATG)	9.8	1.3	0.77	0.18 ± 0.01	0.0229 ± 0.0004	80
d(GTA-GGCT-ATG)	8.9	2.7	0.54	0.217 ± 0.006	0.0163 ± 0.0002	70
d(GTA-CGCG-ATG)	5.5	2.5				
d(GTA-CGCC-ATG)	11.7	2.7		0.162 ± 0.006	0.0060 ± 0.0003	84
d(GTA-CGCA-ATG)	3.1	3.0		0.22 ± 0.02	0.01 ± 0.02	41
d(GTA-CGCT-ATG)	1.9	2.7		0.099 ± 0.06	0.0090 ± 0.009	76
d(GTA-AGCG-ATG)	4.1	2.8	0.61	0.108 ± 0.001	0.048 ± 0.002	35
d(GTA-AGCC-ATG)	6.4	2.4	0.69	0.135 ± 0.003	0.0188 ± 0.0002	74
d(GTA-AGCA-ATG)	1.1	2.7	0.62	0.69 ± 0.04	-	
d(GTA-AGCT-ATG)	< 0.5		0.75	0.38 ± 0.03	0.072 ± 0.005	65
d(GTA-TGCG-ATG)	2.0	2.8	0.67	0.244 ± 0.003	0.0264 ± 0.0005	35
d(GTA-TGCC-ATG)	3.3	2.6	0.62	0.108 ± 0.003	0.0286 ± 0.0005	62
d(GTA-TGCA-ATG)	< 0.5					
d(GTA-TGCT-ATG)	1.8	2.4	0.72	0.83 ± 0.08	0.17 ± 0.02	35

* For the sake of brevity, the complementary strand was not listed.

Table 3. Comparison of Binding and Kinetic Parameters for CHR and MTR at 25 °C.

Oligomers (with CHR)	K (μM^{-1})	*n*/dpx	k_a (min^{-1})	k_{1d} (min^{-1})	k_{2d} (min^{-1})	%
d(ATTA-GGCC-TAAT)	1.5	3.0	0.36	0.092	0.008	44
d(ATTA-CGCG-TAAT)	1.3	2.7	0.39	0.17	0.066	48
d(ATTA-AGCT-TAAT)	< 0.2	-	0.81	1.7	0.25	56
d(ATTA-TGCA-TAAT)	< 0.2	-	-	-	-	-

Oligomers (with MTR)	K (μM^{-1})	*n*/dpx	k_a (min^{-1})	k_{1d} (min^{-1})	k_{2d} (min^{-1})	%
d(ATTA-GGCC-TAAT)	0.4	-	0.13	2.6	0.39	35
d(ATTA-CGCG-TAAT)	0.6	-	0.16	2.7	0.34	34
d(ATTA-AGCT-TAAT)	0.8	-	0.13	0.64	0.16	37
d(ATTA-TGCA-TAAT)	0.2	-	0.18	10	0.5	45